

Epidemiological Outbreaks of *Pneumocystis jirovecii* Pneumonia Are Not Limited to Kidney Transplant Recipients: Genotyping Confirms Common Source of Transmission in a Liver Transplantation Unit

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Over a 5-month period, four liver transplant patients at a single hospital were diagnosed with *Pneumocystis jirovecii* pneumonia (PCP). This unusually high incidence was investigated using molecular genotyping. Bronchoalveolar lavage fluids (BALF) obtained from the four liver recipients diagnosed with PCP were processed for multilocus sequence typing (MLST) at three loci (*SOD*, *mt26s*, and *CYB*). Twenty-four other BALF samples, which were positive for *P. jirovecii* and collected from 24 epidemiologically unrelated patients with clinical signs of PCP, were studied in parallel by use of the same method. *Pneumocystis jirovecii* isolates from the four liver recipients all had the same genotype, which was different from those of the isolates from all the epidemiologically unrelated individuals studied. These findings supported the hypothesis of a common source of contamination or even cross-transmission of a single *P. jirovecii* clone between the four liver recipients. Hospitalization mapping showed several possible encounters between these four patients, including outpatient consultations on one particular date when they all possibly met. This study demonstrates the value of molecular genotyping of *P. jirovecii* isolated from clinical samples for epidemiological investigation of PCP outbreaks. It is also the first description of a common source of exposure to a single *P. jirovecii* clone between liver transplant recipients and highlights the importance of prophylaxis in such a population.

Pneumocystis jirovecii pneumonia (PCP) is an opportunistic fungal infection. Interstitial lung disease and a febrile cough, with or without sputum, are typically observed (1). PCP epidemiology has evolved substantially for the past decade: its incidence was largely reduced in patients infected with human immunodeficiency virus (HIV) after the advent of active antiretroviral therapy (2), whereas it is now a great concern in non-HIV patients, such as those with hematological malignancies (3) and those who have undergone solid organ transplantation (SOT) (4). For instance, in France, its incidence in such non-HIV individuals increased from 0.13 to 0.35 case/10⁵ individuals/year over the 2001–2010 period (5). Regarding the example of renal transplant recipients in England, the number of PCP cases increased 388% from 2006 to 2010, while the number of renal transplantations increased by only 25% (2).

While the exact route of transmission is not fully understood, the results of several studies support an interhuman airborne transmission route (1). Thus, whereas PCP used to occur mainly in the form of isolated cases, several clusters were recently reported, mostly from renal transplant wards (6).

Pneumocystis jirovecii replication cannot be obtained routinely in culture (7), so PCP diagnosis relies on microscopic observation of respiratory samples, mostly bronchoalveolar lavage fluid (BALF) samples, or on molecular detection of *P. jirovecii* DNA by real-time quantitative PCR (qPCR) (1, 8). These techniques are not suitable for investigating the transmission routes of *P. jirovecii* (1, 9) or for epidemiological investigations of a PCP outbreak (10).

Thus, alternative molecular tools (9, 10), such as multilocus sequence typing (MLST), microsatellite genotyping, or PCR-restriction fragment length polymorphism (PCR-RFLP) analysis, have been used to trace strain circulation in the context of hospitals (11–13). Microsatellite genotyping may be more appropriate for detection of mixed infections (14), and PCR-RFLP analysis of the mitochondrial large-subunit rRNA gene (*mtLSU*) alone has appeared to be sufficient for roughly distinguishing between strains (12, 13), but MLST has been shown to be highly reproducible and facilitates comparison of data from different laboratories (11).

Here we report a cluster of four PCP infections that occurred over a 5-month period in our liver transplantation unit. Molecu-

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lar typing demonstrated the genetic similarity of the *P. jirovecii* DNAs collected from the four liver recipients, and epidemiological investigation revealed 10 possible encounter dates.

MATERIALS AND METHODS

Context of the study. Our university hospital has 2,008 beds and includes three main sites dispersed over a few kilometers; the liver transplantation unit is located at the surgical site. Liver transplantation activity started there on 1 January 2011. The immunosuppressive regimen relied on a combination of tacrolimus, mycophenolate mofetil, and prednisone. Until the time of the present study, only two spaced PCP cases, in 2011 and 2012, had previously been diagnosed among 285 liver transplant patients. In contrast, four liver recipients, herein referred to as P1 to P4, were diagnosed with PCP during a 5-month period (25 June 2014 to 13 November 2014).

Biological procedure for routine PCP diagnosis. The diagnostic procedure in our lab relies on both direct examination by conventional methods, using May Grünwald-Giemsa staining (Millipore Merck Chimie SAS, Fontenay-sous-Bois, France) and Uvitex 2B fluorescence (Ciba-Geigy, Basel, Switzerland) (visualization of one cyst is sufficient), and the results of a real-time qPCR that targets the *P. jirovecii* mtLSU gene by using the oligonucleotide primers PjF1 (5'-CTGTTTCCCTTTCGACTATCTACC TT-3') and PjR1 (5'-CACTGAATATCTCGAGGGAGTATGAA-3') and the TaqMan-MGB PjSL probe (5'-6-carboxyfluorescein [FAM]-TCGCA CATAGTCTGATTAT-MGB-3'), as described previously (8). The positive cutoff value for qPCR was set at 35 quantitative cycles (C_q). Each clinical sample was tested in duplicate. Inhibition was assessed with an exogenous positive internal control (universal inhibition control Cy5; Diagenode, Seraing-sur-Meuse, Belgium).

The final diagnosis of PCP infection was systematically approved by physicians, based on clinical examination and medical imaging and supported by the results of the above-mentioned biological tests when at least one of the latter was positive (15).

Study population and biological samples. BALF specimens from the four liver transplant patients (P1 to P4) plus 24 other BALF specimens found positive for *P. jirovecii* between January 2013 and April 2015 (and corresponding to 24 patients [P5 to P28]) were included. The latter samples were collected from individuals with a definitive diagnosis of PCP. These individuals had been hospitalized at different times and in different wards of our institution and were thought to be epidemiologically unrelated to patients P1 to P4. Cases of colonization, i.e., cases with a positive qPCR but a negative direct examination and an absence of clinical and radiological signs of PCP, were not included.

Genotyping of *P. jirovecii* isolates from clinical samples. BALF samples were centrifuged ($1,730 \times g$, 10 min), and each pellet was resuspended in 200 μ l phosphate-buffered saline. DNA was extracted using a QIAmp DNA minikit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. DNA extracts were stored at -20°C until subsequent analysis. PCR was carried out in a 25- μ l final volume, using 5 μ l of 5 \times Q5 reaction buffer (New England BioLabs France, Evry, France), 0.5 μ l of 10 mM (each) deoxynucleoside triphosphates (dNTPs; Promega, Charbonnières, France), 0.25 μ l of 5-U/ μ l Taq Q5 high-fidelity DNA polymerase (New England BioLabs France, Evry, France), and 5 μ l of DNA extract. As previously reported (16, 17), the oligonucleotide primers were used at a final concentration of 0.5 μ M to amplify the following loci: the manganese superoxide dismutase gene (*SOD*), the mitochondrial rRNA gene (mt26S; also referred to as the mtLSU rRNA gene), and the cytochrome *b* gene (*CYB*). A Biometra T3000 (Thermal Gradient Technology, Rochester, NY) apparatus was used under the following conditions: initial denaturation for 30 s at 98°C followed by 40 cycles of hybridization and elongation (10 s at 98°C , 30 s at 60°C , and 30 s at 72°C), with a final elongation step at 72°C for 120 s and an unlimited cooling step at 4°C . Nucleotide sequencing was achieved using a BigDye Terminator, version 1.1, kit (Life Technologies SAS, Saint-Aubin, France), the primers indicated above, and an ABI-3130XL genetic analyzer (Life Technologies

SAS, Saint-Aubin, France). Nucleotide sequences were analyzed using CodonCode Aligner 5.1.5 software (CodonCode Corporation, Centerville, MA) (11, 18). The NCBI GenBank accession numbers for the reference sequences are as follows: AF146753 for *SOD*, M58605 for mt26S, and AF320344 for *CYB*.

Phylogenetic relationships. Phylogenetic relationships between the *P. jirovecii* strains were established according to the unweighted-pair group method using average linkages (UPGMA) by use of MEGA v6.06 software (The Biodesign Institute, Tempe, AZ), with 1,000 bootstrap samples. *Pneumocystis murina* reference sequences (GenBank accession numbers XM_007875480.1, AB626627.1, and XM_007875288.1 for *SOD*, mt26S, and *CYB*, respectively) were used as outgroups.

Transmission map. To study patient movements within the hospital and to identify possible sites of encounter for the four liver transplant recipients with PCP (P1 to P4), dates of outpatient visits and hospitalization were extracted from the medical records in the hospital computing database (Dossier Patient Partagé, Cerner SAS, Paris-La Défense, France).

Statistical analysis. XLStat 2014 (Addinsoft, Paris, France) for Windows (Microsoft, Issy-les-Moulineaux, France) was used for statistical analysis.

Ethics. BALF were stored in the hospital collection (accession no. DC-20100-1216) as approved by the French Ministry of Research. This work was approved by the ethics committee of Tours University Hospital (Espace de Réflexion Ethique, Région Centre, France; approval no. 2015 23). The study registration number (2015_030) was issued by the Technology and Freedom National Committee (Commission de l'Informatique et des Libertés) on 27 May 2015. The study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and complied with BRISQ guidelines (19).

RESULTS

Study population. The four clustered cases of PCP infection (P1 to P4) occurred a mean of 113.8 days after liver transplantation (standard deviation [SD], 33.3 days). The attack rate was estimated to be 9.76% over the study period (25 June 2014 to 13 November 2014), versus 0.82% in the 243 liver recipients from the previous period (1 January 2011 to 25 June 2014). All four patients were males who were more than 50 years old. None had been receiving anti-*P. jirovecii* chemoprophylaxis. Cough was the most prevalent clinical sign. The mean lymphocyte count was below 10^9 cells/liter for each.

The control cohort of unrelated patients (P5 to P28) included HIV-infected individuals, kidney recipients, and patients with autoimmune diseases or cancer. Only three of them were being given anti-*P. jirovecii* primary chemoprophylaxis when *P. jirovecii* was isolated (but with no way of ensuring correct compliance for two individuals prescribed trimethoprim-sulfamethoxazole; the other patient was given aerosols of pentamidine isethionate monthly).

The demographic characteristics of the 28 patients are summarized in Table 1.

***P. jirovecii* genotyping.** MLST results for all patients (P1 to P28) are reported in Table 2. In all, 22 clinical samples (78.6%) were successfully analyzed at the *SOD* locus, 27 (96.4%) at the mt26S locus, and 23 (82.1%) at the *CYB* locus. *P. jirovecii* genotyping was thus fully resolved for the four liver transplant recipients (P1 to P4) and for 18 of the controls. Some haplotypes at certain positions in the *SOD* (T/191), mt26S (4xA/54-57 and C/80), and *CYB* (C/299, A/348, C/362, G/369, C/547, A/675, C/742, and TT/832-33) loci were identical in all samples tested. The mt26S and *CYB* loci both allowed the identification of five distinct genotypes, while only three were demonstrated with the *SOD* locus. Heterozygous allelic positions were observed in five

TABLE 1 Patient characteristics^a

| Characteristic | Value (mean \pm SD or <i>n</i> [%]) (95% CI) | |
|---|--|------------------------------------|
| | Cases (<i>n</i> = 4) | Controls (<i>n</i> = 24) |
| Age (yr) | 55.5 \pm 5.0 (45.2–65.8) | 57.5 \pm 13.9 (49.5–63.3) |
| Male gender | 4 (100.0) (100.0–100.0) | 18 (75.0) (57.7–92.3) |
| Lymphocyte count (10 ⁹ cells/liter) | 0.38 \pm 0.17 (0.00–0.79) | 0.56 \pm 0.32 (0.34–0.78) |
| CD4 ⁺ T-cell count (10 ⁹ cells/liter) | 0.19 \pm 0.20 (0.00–1.97) | 0.11 \pm 0.07 (0.07–0.15) |
| Underlying disease | | |
| Solid tumors | | 4 (16.7) (1.8–31.6) |
| Hematological malignancies | | 1 (4.2) (0.0–12.2) |
| Solid organ transplantation | 4 (100.0) (100.0–100.0) | 4 (16.7) (1.8–37.6) |
| Liver transplantation | 4 (100.0) (100.0–100.0) | |
| Kidney transplantation | | 4 (16.7) (1.8–31.6) |
| HIV | | 11 (45.8) (25.9–65.8) |
| Autoimmune diseases | | 3 (12.5) (0.0–25.7) |
| Miscellaneous other diseases | | 1 (4.2) (0.0–12.2) |
| Clinical course | | |
| Fever ($\geq 38^{\circ}\text{C}$) | 2 (50.0) (1.0–99.0) | 17 (70.8) (52.6–89.0) |
| Cough | 4 (100.0) (100.0–100.0) | 19 (79.2) (62.9–95.4) |
| Sputum | 2 (50.0) (1.0–99.0) | 6 (25.0) (7.7–42.3) |
| Dyspnea | 2 (50.0) (1.0–99.0) | 19 (79.2) (62.9–95.4) |
| Chest pain | | 2 (8.3) (0.0–19.4) |
| Asthenia | 3 (75.0) (32.6–100.0) | 19 (79.2) (62.9–95.4) |
| Headaches | 1 (25.0) (0.0–67.4) | 1 (4.2) (0.0–12.2) |
| Radiological findings ^b | | |
| Interstitial lung disease | 4 (100.0) (100.0–100.0) | 19 (79.2) (62.9–95.4) |
| Alveolar consolidation | | 2 (8.3) (0.0–19.4) |
| Micronodules | | 4 (16.7) (1.8–31.6) |
| Anti- <i>P. jirovecii</i> primary chemoprophylaxis ^c | 0 (0.0) (0.0–0.0) | 3 (12.5) (0.0–25.7) |
| Anti- <i>P. jirovecii</i> curative therapy ^b | | |
| TMP-SMX | 4 (100.0) (100.0–100.0) | 23 (95.8) (87.8–100.0) |
| Atovaquone | 1 (75.0) (32.6–100.0) | 7 (29.2) (11.0–47.4) |
| Pentamidine isethionate | | 1 (4.2) (0.0–12.2) |
| Total duration of PCP (days) | 21.0 \pm 0.0 (21.0–21.0) | 23.1 \pm 4.4 (20.3–26.0) |
| Clinical outcome (survival) | 2 (50.0) (1.0–99.0) | 19 ^d (82.6) (67.2–98.1) |

^a Abbreviations: 95% CI, 95% confidence interval; SD, standard deviation; TMP-SMX, trimethoprim-sulfamethoxazole.

^b Associations are possible.

^c There is no way to determine whether compliance was satisfactory.

^d One patient was lost to follow-up.

clinical samples (17.9%), consistent with mixed *P. jirovecii* infections.

Altogether, MLST evidenced 19 complete multilocus genotypes. The 18 *P. jirovecii* strains from epidemiologically unrelated patients each corresponded to different genotypes (B to N). In stark contrast, the *P. jirovecii* strains from the four liver recipients all exhibited the same single genotype (genotype A), supporting either a common source of contamination or cross-contamination between patients. It should be noted that patients P15, P16, P18, and P21 displayed the same allele for the mt26S locus as that for patients P1 to P4, but because amplification of the *SOD* and *CYB* sequences was unsuccessful for their strains, it was not possible to determine whether the strains belonged to the same clone as that involved in the four liver recipients.

Phylogenetic relationships. Phylogenetic analysis (Fig. 1) indicated that the epidemiologically unrelated *P. jirovecii* strains were heterogeneously dispersed, whereas the strains from patients P1 to P4 were tightly clustered (supported by bootstrap values of $\geq 90\%$).

Hospitalization mapping. The movements of the four liver recipients (P1 to P4) within the university hospital are summarized in Fig. 2. This transmission map revealed 10 possible encounter dates, including 7 dates for visits to the outpatient clinic of the liver transplantation unit (arrows 2 and 5 to 10). Patient P1 was first diagnosed as positive for PCP 73 days after undergoing liver transplantation. Twenty-four weeks before PCP diagnosis, patient P2 encountered P1 during hospitalization in the liver transplantation unit, while he was in his early posttransplantation period. Patient P3 may have met P1 five

TABLE 2 Allelic profiles of the *SOD*, *mt26S*, and *CYB* regions in all the *P. jirovecii* strains studied^a

| Patient no. | Date of diagnosis (mo-day-yr) | SOD locus | | | | mt26S locus | | | | CYB locus | | | | | | | | | | | | CYB genotype (1,2) | Multilocus genotype ^{e,f} | | | |
|-------------|-------------------------------|-------------------------------|-----|-----|--------------------|----------------------------------|----|-----|----------------------|----------------------------------|--------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------------------|------------------------------------|--------------------|--------------------|---|
| | | mt at position ^b : | | | SOD genotype (1,2) | mt at position(s) ^c : | | | mt26S genotype (1,3) | mt at position(s) ^d : | | | | | | | | | | | | | | | | |
| | | 110 | 191 | 215 | | 54-57 | 80 | 85 | | 248 | 288 | 279 | 299 | 348 | 362 | 369 | 516 | 547 | 566 | 675 | 742 | | | 832-833 | 838 | |
| P1 | 06-25-14 | C | T | T | SOD1 | 4×A | C | C | T | A | Allele 3 | C | C | A | C | G | C | C | C | A | C | C | TT | T | CYB2 | A |
| P2 | 08-08-14 | C | T | T | SOD1 | 4×A | C | C | T | A | Allele 3 | C | C | A | C | G | C | C | C | A | C | TT | T | CYB2 | A | |
| P3 | 09-22-14 | C | T | T | SOD1 | 4×A | C | C | T | A | Allele 3 | C | C | A | C | G | C | C | C | A | C | TT | T | CYB2 | A | |
| P4 | 11-13-14 | C | T | T | SOD1 | 4×A | C | C | T | A | Allele 3 | C | C | A | C | G | C | C | C | A | C | TT | T | CYB2 | A | |
| P5 | 01-09-13 | C | T | T | SOD1 | 4×A | C | C | T | A | Allele 8 | T | C | A | C | G | T | C | C | A | C | TT | C | CYB5 | B | |
| P6 | 01-17-13 | C | T | T/C | Mixed (SOD1/ SOD4) | 4×A | C | C/T | C | A | Mixed (allele 2/8) | T | C | A | C | G | T | C | C | A | C | TT | C | CYB5 | Mixed ^f | |
| P7 | 03-08-13 | C | T | C | SOD4 | 4×A | C | C | T | A | Allele 3 | T | C | A | C | G | C | C | C | A | C | TT | C | CYB8 | C | |
| P8 | 04-10-13 | T | T | C | SOD2 | 4×A | C | C | C | G | Allele 1 | C | C | A | C | G | C | C | C | A | C | TT | C | CYB1 | D | |
| P9 | 04-23-13 | T/C | T | C/T | Mixed (SOD2/ SOD1) | 4×A | C | A/T | C | A | Mixed (allele 7/8) | C | C | A | C | G | C/T | C | C | A | C | TT | C | Mixed (CYB1/ CYB6) | Mixed ^f | |
| P10 | 05-10-13 | C | T | T | SOD1 | 4×A | C | C | T | A | Allele 3 | C | C | A | C | G | C | C | C | A | C | TT | C | CYB1 | E | |
| P11 | 07-17-13 | C | T | T | SOD1 | 4×A | C | A | C | A | Allele 7 | T | C | A | C | G | C | C | C | A | C | TT | C | CYB8 | F | |
| P12 | 07-29-13 | T | T | C | SOD2 | 4×A | C | C | C | A | Allele 2 | T | C | A | C | G | C | C | C | A | C | TT | C | CYB8 | G | |
| P13 | 09-12-13 | / | / | / | ND | / | / | / | / | / | ND | / | / | / | / | / | / | / | / | / | / | / | / | ND | ND | |
| P14 | 09-12-13 | C | T | T | SOD1 | 4×A | C | C | C | A | Allele 2 | C | C | A | C | G | C | C | C | A | C | TT | T | CYB2 | H | |
| P15 | 02-12-14 | / | / | / | ND | 4×A | C | C | T | A | Allele 3 | / | / | / | / | / | / | / | / | / | / | / | / | ND | ND ^s | |
| P16 | 04-18-14 | / | / | / | ND | 4×A | C | C | T | A | Allele 3 | / | / | / | / | / | / | / | / | / | / | / | / | ND | ND ^s | |
| P17 | 03-25-14 | C/T | T | T/C | Mixed (SOD1/ SOD2) | 4×A | C | C/T | T/C | A | Mixed (allele 3/8) | T/C | C | A | C | G | C/T | C | C | A | C | TT | C | Mixed (CYB8/ CYB6) | Mixed ^f | |
| P18 | 03-25-14 | / | / | / | ND | 4×A | C | C | T | A | Allele 3 | / | / | / | / | / | / | / | / | / | / | / | / | ND | ND ^s | |
| P19 | 03-27-14 | / | / | / | ND | 4×A | C | C | T | A | Allele 3 | C | C | A | C | G | T | C | C | A | C | TT | C | CYB6 | ND | |
| P20 | 05-05-14 | T/C | T | C/T | Mixed (SOD2/ SOD1) | 4×A | C | T | C | A | Allele 8 | C | C | A | C | G | C/T | C | C | A | C | TT | C | Mixed (CYB1/ CYB6) | Mixed ^f | |
| P21 | 05-15-14 | / | / | / | ND | 4×A | C | C | T | A | Allele 3 | / | / | / | / | / | / | / | / | / | / | / | / | ND | ND ^s | |
| P22 | 10-07-14 | C | T | T | SOD1 | 4×A | C | T | C | A | Allele 8 | C | C | A | C | G | T | C | C | A | C | TT | C | CYB6 | I | |
| P23 | 10-21-14 | C | T | T | SOD1 | 4×A | C | T | C | A | Allele 8 | T | C | A | C | G | C | C | C | A | C | TT | C | CYB8 | J | |
| P24 | 01-06-15 | C | T | T | SOD1 | 4×A | C | A | C | A | Allele 7 | T | C | A | C | G | T | C | C | A | C | TT | C | CYB5 | K | |
| P25 | 01-06-15 | T | T | C | SOD2 | 4×A | C | C | T | A | Allele 3 | C | C | A | C | G | C | C | C | A | C | TT | C | CYB1 | L | |
| P26 | 03-16-15 | T | T | C | SOD2 | 4×A | C | C | T | A | Allele 3 | T | C | A | C | G | C | C | C | A | C | TT | C | CYB8 | M | |
| P27 | 03-23-15 | C | T | T | SOD1 | 4×A | C | A | C | A | Allele 7 | C | C | A | C | G | C | C | C | A | C | TT | C | CYB1 | N | |
| P28 | 04-08-15 | C/T | T | T/C | Mixed (SOD1/ SOD2) | 4×A | C | C/T | T/C | A | Mixed (allele 3/8) | C | C | A | C | G | C | C | C | A | C | TT | C | CYB1 | Mixed ^f | |

^a The four clustered cases of *Pneumocystis jirovecii* pneumonia (P1 to P4) in liver recipients are underlined. Abbreviations: A, adenine; CYB, cytochrome *b* gene; C, cytosine; —, nucleotide deletion; /, sequencing failure; G, guanine; *mt26S*, mitochondrial rRNA gene; NA, not available; ND, unspecified; nt, nucleotide; *SOD*, manganese superoxide dismutase gene; T, thymine.

^b Numbering is according to the *SOD* reference sequence (GenBank accession no. AF146753).

^c Numbering is according to the *mt26S* reference sequence (GenBank accession no. M58605).

^d Numbering is according to the *CYB* reference sequence (GenBank accession no. AF320344).

^e The multilocus genotype was determined from the combination of the three loci considered together. Two multilocus genotypes were similar if the combinations of the three alleles were the same.

^f More than one *P. jirovecii* genotype (for each patient sample displaying a mixed genotype, the putative combination of genotypes is given, based on the hypothesis that the *P. jirovecii* genome is haploid).

^g Since only *mt26S* (*SOD* and *CYB* are single-copy genes) was successfully amplified for each, it was impossible to determine whether the multilocus genotype was or was not the same as that of the isolates from patients P1 to P4.

However, identity with the sequences of the isolates from patients P1 to P4 was in no case total (99%), and there was one additional nucleotide gap. The four concerned patients (P15, P16, P18, and P21) were all kidney recipients, and PCP occurred over a 3-month period for them. They were not hosted in the same geographical site of our hospital as the four liver recipients with PCP and consequently never met them.

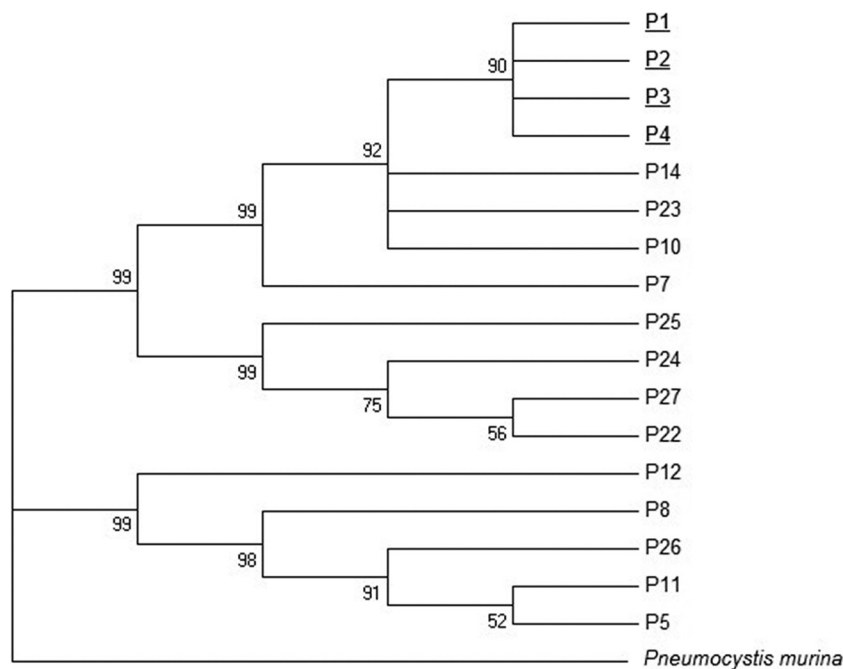


FIG 1 Phylogenetic tree of *P. jirovecii* strains based on the nucleotide sequences of their *SOD*, *mt26S*, and *CYB* regions, according to the UPGMA method. The four clustered cases of *Pneumocystis jirovecii* pneumonia (P1 to P4) which occurred concomitantly in liver recipients are underlined. All samples with mixed genotypes and those with amplification failures were excluded from this representation. The outgroup sequences are from *Pneumocystis murina* and were referenced with the following NCBI GenBank accession numbers: [XM_007875480.1](#) (*SOD*), [AB626627.1](#) (*mt26S*), and [XM_007875288.1](#) (*CYB*). The UPGMA method enables matching of strains with the most similar sequences to each other, regardless of the rate of evolution. The minimum bootstrap value was set at 1,000. High bootstrap scores demonstrate the reliability of embranchment.

times and P2 twice (arrows 2, 4 to 7, and 9) before PCP diagnosis, including five encounters between days 27 and 119 after transplantation. Patient P4 may have encountered P3 (arrow 3) and P2 (arrow 8) 76 days and 10 days before transplantation,

respectively. P4 may also have encountered P1, P2, and P3 in the outpatient clinic of the liver transplantation unit 33 days after the graft, and also P1 again, 14 days later (arrows 9 and 10).

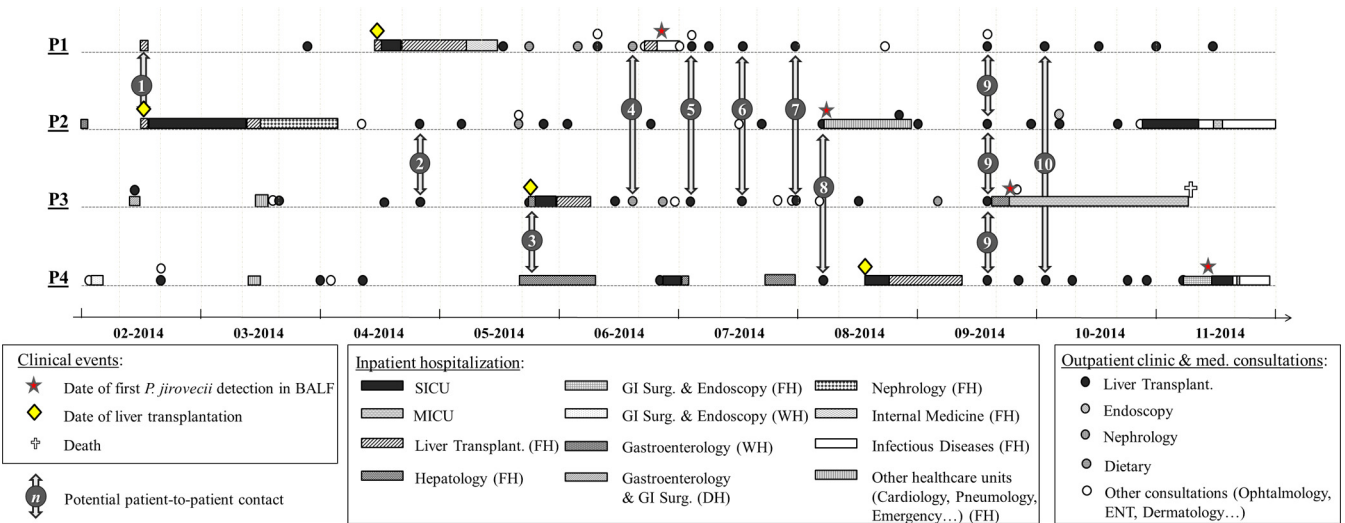


FIG 2 Transmission mapping of *P. jirovecii* among liver transplant recipients. The x axis shows dates (month-year), and the y axis shows patient numbers. The four clustered cases of *Pneumocystis jirovecii* pneumonia (P1 to P4) which occurred concomitantly in liver recipients are underlined. They were all due to strains with no mutation in the dihydropteroate synthetase (DHPS) gene, which is debated to be involved in resistance to trimethoprim-sulfamethoxazole. Numbered arrows denote the 10 encounter dates compatible with interindividual *P. jirovecii* transmission. Arrows 9 indicate possible contacts for all four liver transplant patients (18 September 2014) in the outpatient clinic of liver transplantation. Other arrows point to dates of possible one-on-one encounters. Abbreviations: BALF, bronchoalveolar lavage fluid; DH, day of hospitalization; ENT, ear-nose-throat; FH, full hospitalization; GI, gastrointestinal; med., medical; MICU, medical intensive care unit; SICU, surgical intensive care unit; Surg., surgery; Transplant., transplantation; WH, week of hospitalization.

DISCUSSION

Pneumocystis jirovecii is an opportunistic fungus with strict human host specificity. It is responsible for a severe respiratory disease, *Pneumocystis jirovecii* pneumonia (PCP) (1, 20). One potential route of infection is currently believed to be patient-to-patient transmission mediated by airborne droplets (1), and the hypothesis related to contamination from an environmental source has largely been rejected (9, 10).

Previously reported PCP outbreaks in SOT patients generally occurred in kidney recipients, and later after transplantation (6, 21). In contrast, PCP is thought to be a rare event in liver recipients: in older reports, the overall incidence of PCP was around 1% (4, 22). This observation may be supported by the fact that liver transplantations overall are practiced less commonly than kidney transplantations (for instance, 285 versus 480, respectively, in our study over the period from 1 January 2011 to 13 November 2014). Interestingly, in the present study, four PCP infections were unusually diagnosed in liver recipients within a 5-month period in 2014. We analyzed the reasons for this sudden cluster, as the diagnostic procedures and immunosuppressive regimens had remained similar over the past 5 years and thus could not explain this emergence. Except for one patient of the cluster, the individuals with PCP were not more immunocompromised than and their respective underlying disease was not more serious than that of other liver recipients during the same period. It should be noted that our institution did not apply systemic anti-PCP chemoprophylaxis for liver transplant patients because the incidence of this opportunistic infection had always remained lower than 0.01 (two cases among 285 patients over 4 years). Indeed, although it has been shown that the risk of acquiring PCP may increase in SOT recipients not taking effective prophylaxis (6, 23, 24), routine prophylaxis is recommended only for centers with an incidence of PCP of at least 3 to 5% among transplant recipients (25). In such health care settings, trimethoprim-sulfamethoxazole remains the drug of choice (80 to 160/400 to 800 mg orally daily or three times weekly [grade I] for at least 6 to 12 months posttransplantation [grade III]) (26). Some authors also recommend strict hospital segregation of immunocompromised individuals with PCP (patients at risk of PCP should not share a hospital room with a patient with PCP) and the systematic use of face-mask filtering to prevent airborne *P. jirovecii* transmission among infected patients (18). Nevertheless, clinical data to support this recommendation in practice are insufficient (27), and consequently the issue is still the subject of debate. Because the outbreak spontaneously stopped in our hospital by the end of 2014, it was considered only a sporadic event that should not force changes in our clinical practices. No subsequent PCP case has been reported so far.

Nonetheless, we attempted to explain the epidemiology of this PCP cluster. Globally, the epidemiology of this disease has always been difficult to study (21, 23, 28, 29), mainly due to the lack of easy-to-perform methods of culture and because alternative powerful and informative techniques have not been available in all microbiology laboratories (30). However, MLST is now used widely to investigate nosocomial PCP outbreaks and is generally considered the gold standard (6, 10, 11, 18, 31). Recently, Maitte et al. showed that a simple scheme relying on three loci, *SOD*, *mt26S*, and *CYB*, provides sufficient discrimination to be used reliably for epidemiological investigations of PCP outbreaks (11). Our MLST analysis based on this approach indeed highlighted substantial

nucleotide polymorphism in the *P. jirovecii* genome and evidenced several *P. jirovecii* infections involving more than one strain (11, 18, 31, 32). In addition, our findings clearly support the conclusion that liver transplant patients P1 to P4 were all infected by a single genotype of *P. jirovecii*. This conclusion seems robust because, as shown by the phylogenetic tree, data for the three loci combined were sufficient to discriminate between 24 unrelated *P. jirovecii* isolates (10). To our knowledge, this is the first genotypic demonstration of clustered PCP limited only to liver transplant patients (24), whereas numerous studies have reported similar findings for kidney recipients (6, 10, 18), especially in France (10). Thus, in accordance with recent works regarding SOT (26), our study suggests that the use of prophylaxis as widely practiced in most centers of kidney transplantation (22, 26) should also be considered thoroughly for liver recipients.

Our mapping of patient movements in the hospital also supports the hypothesis of nosocomial acquisition and patient-to-patient transmission of *P. jirovecii* among liver transplant patients, at least for patients P2, P3, and P4 (6, 10, 18). Indeed, cross-transmission may have been facilitated in our institution due to the distance between the different wards in charge of those patients leading to multiple transfers and the frequentation of diverse waiting areas of outpatient clinics. The demonstration of interpatient transmission is hampered (9), however, because the exact incubation time of PCP remains unknown, as does the time of *P. jirovecii* excretion into the environmental air (8). In addition, colonized patients without symptoms of PCP may also have played a role in this transmission, though their pulmonary fungal burdens are known to be lower (8). In this study, the periods from encounter with a proven index case to PCP diagnosis were between 4 and 172 days. Due to the retrospective design of this study, we cannot exclude the possibility that the transmission of *P. jirovecii* may have involved asymptomatic subjects, such as transiently colonized nurses and doctors (33). Very few studies have been able to provide molecular evidence that colonized individuals may serve as infectious sources of *P. jirovecii* (10). In contrast, there was no way to assume a common source of contamination by an inert material, such as a bronchoscope, since the BAL procedures for the four patients were not performed in the same health care department and were performed on different days (our sterilizing procedures are scheduled daily).

Conclusions. Our study shows that PCP remains a public health issue and that effective preventive measures are required for immunocompromised patients, especially for liver transplant recipients. We also demonstrated the clinical and epidemiological value of *P. jirovecii* genotyping by a simple MLST scheme for investigations of nosocomial outbreaks.

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We have no conflicts of interest to disclose.

We were all involved in the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and the final approval of the version to be submitted.

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